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Vitamin B-12–fortified toothpaste improves vitamin status in vegans: a 12-wk randomized placebo-controlled study[1,2]

*Anne-Kathrin Siebert,[3,7] Rima Obeid,[4,7] Stine Weder,[3] Hussain M Awwad,[5] Andreas Sputtek,[6] Juergen Geisel,[5] and Markus Keller[3]**

[3]Institute of Alternative and Sustainable Nutrition, Biebertal, Giessen, Germany; [4]Aarhus Institute of Advanced Studies, Aarhus University, Aarhus, Denmark; [5]Department of Clinical Chemistry and Laboratory Medicine, Saarland University Hospital, Homburg, Saarland, Germany; and [6]Center for Laboratory Medicine and Microbiology, Essen, Germany

ABSTRACT

Background: The oral application of vitamin B-12 may prevent its deficiency if the vitamin is absorbed via the mucosal barrier.

Objectives: We studied the effect of the use of a vitamin B-12–fortified toothpaste on vitamin-status markers in vegans and assessed the efficiency of markers in the identification of vitamin-augmentation status.

Design: In this 12-wk, double-blinded, randomized, placebo-controlled study, 76 vegans received either a placebo (n = 34) or vitamin B-12 (n = 42) toothpaste. Sixty-six subjects (n = 30 in the placebo arm; n = 36 in the vitamin B-12 arm) completed the intervention. Serum and plasma concentrations of vitamin B-12, holotranscobalamin, total homocysteine (tHcy), and methylmalonic acid (MMA) were measured before and after the intervention.

Results: Both postintervention concentrations of vitamin B-12 and holotranscobalamin and their changes over 12 wk were higher in the vitamin B-12 group (mean ± SD change: 81 ± 135 pmol/L for vitamin B-12 and 26 ± 34 pmol/L for holotranscobalamin) than in the placebo group (−27 ± 64 and −5 ± 17 pmol/L, respectively) after adjustment for baseline concentrations. Postintervention concentrations of MMA and their changes differed significantly between groups (MMA changes: −0.169 ± 0.340 compared with −0.036 ± 0.544 μmol/L in vitamin B-12 and placebo groups, respectively; $P < 0.001$). After adjustment for baseline tHcy, postintervention concentrations of tHcy tended to be lower ($P = 0.051$), and the changes in tHcy (−0.7 ± 4.4 compared with 2.0 ± 5.6 μmol/L, respectively) were greater in the vitamin B-12 group than in the placebo group. Changes in vitamin B-12 markers were more prominent in vegans who reported that they had not taken vitamin B-12 supplements.

Conclusion: Vitamin B-12 that is applied to the oral cavity via toothpaste enters the circulation and corrects the vitamin B-12 markers in the blood of vegans who are at higher risk of vitamin B-12 deficiency. This trial was registered at clinicaltrials.gov as NCT02679833.
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Keywords: deficiency, holotranscobalamin, methylmalonic acid, supplementation, vegans, vitamin B-12

INTRODUCTION

Vitamin B-12 deficiency is common in individuals with low intake of animal-based foods and in people with impaired vitamin absorption. In individuals with a normal absorption capacity, deficiency can be prevented via oral vitamin supplementation in small doses and intake of various vitamin B-12–supplement variants such as oral tablets, drops, sublingual (1), and nasal sprays (2, 3). Studies have reported a sharp increase in plasma vitamin B-12 concentration shortly after its nasal application although, to our knowledge, the mechanisms of vitamin B-12 absorption through the nasal mucosal membrane are unknown. The nasal (4) and salivary (5) glands produce the vitamin B-12–binding protein haptocorrin that has no clear biological function. The human submandibular gland also produces haptocorrin in the mucous-secreting acini and intercalated ducts (6). Sublingual and nasal vitamin B-12 supplements that have been tested thus far have contained supraphysiologic doses of the vitamin (500–1500 μg) that are likely to cross the membrane via passive diffusion or even swallowing to be later absorbed via intrinsic factors. The introduction of vitamin B-12 into the oral cavity at lower doses via a nonpharmaceutical vehicle, such as toothpaste, may serve as an alternative administration route. However, to our knowledge, the efficacy of this route in correcting vitamin B-12 deficiency has not been tested.

Vegans do not consume any animal products and are, therefore, a classic risk group for vitamin B-12 deficiency (7–9). Although awareness regarding vitamin B-12 deficiency has increased in vegans, many vegans still either do not use any supplements or have reported irregular use (7, 9). Vitamin B-12 and holotranscobalamin concentrations are low, and methylmalonic acid (MMA)[8] (7) and total homocysteine (tHcy) concentrations are elevated, in subjects with vitamin B-12 deficiency (10, 11). Abnormal concentrations of

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[2] Supplemental Figures 1–4 and Supplemental Tables 1 and 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://ajcn.nutrition.org.

[7] These authors share first authorship.

*To whom correspondence should be addressed. E-mail: markus.keller@ifane.org.

[8] Abbreviations used: cB-12, combined vitamin B-12 indicator; MMA, methylmalonic acid; ROC, receiver operating characteristic; tHcy, total homocysteine; 4cB-12, 4 markers of combined vitamin B-12 indicator.

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these biomarkers are suggestive of a subclinical deficiency even in the absence of clear symptoms (12). The use of stepwise algorithms (13, 14) or an evaluation of a direct combination of available markers (15) has been suggested for the diagnosis of vitamin B-12 deficiency; however, the efficiency of these methods for the detection of vitamin B-12 status has not been well studied.

The identification of alternative, efficacious routes of vitamin B-12 supplementation is an attractive approach to encourage the prevention of deficiency without the need for therapeutic doses or oral pills. Thus, we investigated the effect of a vitamin B-12–fortified toothpaste on the vitamin B-12 status of vegans during a 12-wk usage period. Changes in vitamin B-12 markers were used as outcome measures to evaluate the efficacy of this administration route.

METHODS

Subjects and settings

We recruited vegans at 4 study centers in Berlin, Essen, Giessen, and Homburg (Germany) between November 2014 and March 2015. Seventy-six vegans were recruited during visit 1, and 66 of the vegans returned to the study centers for a second visit after 12 wk (visit 2). The study was conducted in accordance with the ethical principles stated in the Declaration of Helsinki. The study protocol was reviewed and approved by the Ethics Committee of the Faculty of Medicine at Giessen University, and all participants provided written informed consent. This trial was registered at clinicaltrials.gov as NCT02679833.

Study design

The trial was a double-blinded, randomized, placebo-controlled study. Eligible candidates were identified via an online survey that was aimed at recruiting apparently healthy men and women aged between 18 and 50 y who had followed a vegan diet ≥5 y or a vegan diet for the past 2 y after following a vegetarian diet before that. The survey further aimed to exclude all individuals who had diseases or conditions that are known to be associated with vitamin B-12 deficiency, had received high-dose cyanocobalamin (≥1 mg) treatment, or had conditions that influence vitamin B-12 biomarkers such as liver and renal diseases. Other exclusion criteria included pregnancy, lactation, anemia, malabsorption, and diseases of the gastrointestinal tract, the chronic use of medications other than oral contraceptives, and the regular use of vitamin B-12–fortified toothpaste before the study. The use of over-the-counter vitamin B-12–containing supplements was not an exclusion criterion but had to be reported (as yes or no). The survey was based on self-reported information that was not verified by a medical examination.

A total of 2010 people completed the online questionnaire, of whom 123 individuals fulfilled the inclusion criteria and were invited to the study centers (visit 1). Of 99 vegans who accepted the invitation and were randomly assigned (n = 48 in the placebo arm; n = 51 in the vitamin B-12 arm), 23 individuals did not attend visit 1 (**Supplemental Figure 1**). Blood samples were collected from 76 participants who attended visit 1 (n = 34 in the placebo arm; n = 42 in the vitamin B-12 arm). A total of 66 participants (n = 30 in the placebo arm; n = 36 in the vitamin B-12 arm) returned after 12 wk of intervention (visit 2). Analyses of baseline data included all 76 subjects who attended visit 1 and provided blood samples (i.e., the intention-to-treat population). Between-group comparisons at visit 2 as well as within-individual comparisons were performed with the use of available data (per protocol); no imputation was conducted.

The random assignment was performed with the use of the Microsoft Excel RAND function (Microsoft). Three tubes of toothpaste (placebo- or vitamin B-12–containing tubes) were provided to each participant during visit 1. The placebo and vitamin B-12-containing toothpastes were identical in appearance, smell, and taste. The principle investigator (MK) was aware of the coding, but A-KS was mainly responsible for the randomization step (supervised by MK). The participants and the staff members who were responsible for measuring the biomarkers and accessing the study outcome were blinded to the random assignment until the end of the study period.

Participating subjects were asked to maintain their usual lifestyles and to abstain from taking new supplements or the use of fortified foods. The use of vitamin B-12–containing supplements was reported by 53 of 76 vegans [n = 22 in the placebo arm (64.7%), and n = 31 in the vitamin B-12 arm (73.7%); P = 0.456]. The information on vitamin B-12–containing supplements covered any use and frequency ranging from daily to yearly. Subjects who reported regular consumption of vitamin B-12 were asked to maintain the same behavior for the entire study duration. We rationalized that the vitamin B-12–depletion that would have resulted from supplement discontinuation may have opposed any expected active intervention–related augmentation and, thus, may have biased the results toward the null.

Participants were instructed to use the toothpaste 2 times/d for 2 min at a time. Compliance was controlled by the completion of a written protocol that included the type of toothbrush used (i.e., manual or electric) and the time and duration of brushing over the 12-wk period. The remaining unused toothpaste was returned at the end of the study period, and its weight was used as a further measure of compliance.

Estimation of the amount of vitamin B-12 delivered to the oral cavity daily

We estimated the amount of the toothpaste that could fit on the head of a manual toothbrush (mean ± SD: 1.48 ± 0.15 g) or an electrical toothbrush (0.65 ± 0.06 g) in 10 repeated weighing experiments. The vitamin B-12–fortified toothpaste used in our study contained 100 μg cyanocobalamin/g (Logocos Naturkosmetik AG). This amount was verified by water extraction and the measurement of vitamin B-12 with the use of an electrochemiluminescence immunoassay (Cobas autoanalyzer; Roche Diagnostics GmbH). Thus, the average amount of cyanocobalamin that was delivered into the oral cavity was 130 or 290 μg/d for the 2 brushing sessions with the use of an electrical or manual toothbrush, respectively. However, the absorbed amount of cyanocobalamin was not known.

Blood sampling and biochemical analyses

Fasting blood samples (≥8-h fasting period) were collected in dry (for serum) and EDTA-K^+–containing tubes (for plasma) during the 2 visits. Blood samples were centrifuged, and the serum and plasma samples were separated within a maximum of 40 and

30 min, respectively. Samples from the first visit were stored at −70°C until the second visit. Samples were analyzed after completion of the intervention period, and all samples from a participant were analyzed in the same run to minimize variations.

Serum vitamin B-12 and holotranscobalamin (16) concentrations were analyzed at the Labordiagnostik Mittelhessen with the use of the electrochemiluminescence immunoassay (Cobas; Roche Diagnostics GmbH) and chemiluminescence microparticle immunoassay (Architect; Abbott), respectively. tHcy and MMA concentrations were measured at the Central Laboratory of the Saarland University Hospital; plasma tHcy was measured with the use of chemiluminescence microparticle immunoassay (Architect; Abbott Laboratories); and serum MMA was measured with the use of a gas chromatography–mass spectrometry method. The day-to-day coefficients of variations of the analytical methods were all <6%.

We calculated the 4 markers combined vitamin B-12 indicator (4cB-12) status from concentrations of holotranscobalamin, vitamin B-12, tHcy, and MMA according to published equations (adjusted for age) (15, 17). Scores of 4cB-12 were classified into the following 5 categories: probable vitamin B-12 deficiency, possible vitamin B-12 deficiency, low vitamin B-12, vitamin B-12 adequacy, and elevated vitamin B-12 (15).

Sample size and study power

Primary outcomes of the study were differences in the concentrations of holotranscobalamin, vitamin B-12, tHcy, and MMA between the placebo and vitamin B-12 study arms after 12 wk of intervention. The sample-size estimation was based on the change in MMA concentration, which is the functional and more-specific marker of vitamin B-12 status. We predicted a minimum 30% posttreatment difference in the mean MMA between vitamin B-12 and placebo arms. This estimate was based on our earlier studies on vitamin B-12–supplemented vegetarians compared with non-supplemented vegetarians (7). To detect this estimated posttreatment difference in the serum MMA concentration between the 2 study arms, we calculated a group size of 35 participants/arm. The calculation was based on the assumptions that the mean ± SD baseline MMA concentration was 0.400 ± 0.300 μmol/L and that the treatment would cause a 30% difference (or 0.120 μmol/L) in MMA between the arms (80% power with $\alpha = 0.05$). In the 66 participants who completed the study (both visits 1 and 2), a 20% difference in the mean MMA concentration was observed between the 2 arms at baseline. To detect a 50% difference in the mean MMA concentration after the intervention, 27 subjects/arm were required for 80% power, or 35 subjects/arm were required for 90% power (both with $\alpha = 0.05$). Therefore, our study was powered to detect ∼50% differences in posttreatment mean MMA concentrations between the 2 study arms with ≥80% power. The sample size was also sufficient to detect differences in vitamin B-12 and holotranscobalamin concentrations between the 2 arms because these markers are known to show a stronger response to vitamin supplementation than does MMA (18–21).

Statistical analyses

All study data were analyzed with the use of SPSS software (version 24; IBM). Continuous variables were tested for the normality of distribution with the use of the Kolmogorov-Smirnov test with Lilliefors significance correction. All variables were non-normally distributed and are expressed as geometric means ± SDs (median; IQR). Changes in markers between visits 1 and 2 are presented as means ± SDs. A general linear model univariate ANOVA was used to test differences in continuous variables between any 2 independent groups (**Tables 1–3**). A general linear model univariate ANOVA for repeated measurements was used to test within-individual changes (Table 3).

The within-individual changes in markers of vitamin B-12 status during the study period were calculated by subtracting their baseline (visit 1) concentrations from their concentrations at the end of the study (visit 2). These changes were compared between the 2 study arms with the use of a univariate ANOVA. Because differences in baseline concentrations of these markers may affect their response to treatment (study outcome) (**Supplemental Figure 2**), we adjusted all comparisons of concentrations of vitamin B-12 markers and their changes between the study arms for their baseline concentrations as recommended by Bland et al. (22). Further, we conducted subgroup analyses to access the response to treatment according to vitamin B-12–supplement usage (**Supplemental Figure 3**).

Differences in the distribution of categorical variables between the 2 arms were tested with the use of a chi-square test. Correlations between continuous variables were studied with the use of Spearman's test (**Supplemental Table 1**, **Supplemental Figure 4**).

A receiver operating characteristic (ROC) curve analysis was applied to test the abilities of the different markers to differentiate between the vegan participants at week 12 according to their treatment allocations. The AUC was used to compare the performances (sensitivity and specificity) of postintervention markers. All tests were 2-sided, and P values < 0.05 were considered statistically significant, whereas $P > 0.05$ but <0.10 were considered to indicate a tendency.

RESULTS

Characteristics of study population

Table 1 shows the main characteristics of participants according to their treatment allocations. The study arms did not differ significantly according to the mean age or BMI, the proportion of women participants or vitamin-supplement users, and the duration of the vegan diet.

Vitamin B-12–supplement users and nonusers did not differ in age, the proportion of women, or the duration of the vegan diet (Table 2). Compared with vegans who reported no supplement use, vegans who used supplements had higher concentrations of vitamin B-12 (geometric mean: 135 compared with 194 pmol/L, respectively) at baseline. In addition, in vegans who reported no supplement use, concentrations of holotranscobalamin tended to be lower (22 compared with 33 pmol/L, respectively), whereas concentrations of tHcy (15.6 compared with 10.3 μmol/L, respectively) and MMA (0.438 compared with 0.288 μmol/L, respectively) were higher. At baseline, elevated tHcy (>12.0 μmol/L) and MMA (>0.270 μmol/L) and low holotranscobalamin (<35 pmol/L) were observed in 28 (36.8%), 38 (50.0%), and 45 (59.2%) of 76 vegans, respectively, with a significantly lower prevalence in supplement users than in nonusers. Thirty-two of 76 vegans (42.1%) exhibited both elevated MMA and lowered

TABLE 1
Main characteristics of participants as randomly assigned

	All subjects (n = 76)	Placebo (n = 34)	Vitamin B-12 toothpaste (n = 42)	P
Age, y	29.4 ± 7.1 (29.0; 10.0)[1]	29.8 ± 8.0 (29.0; 10.0)	29.1 ± 6.3 (28.8; 10.0)	0.525[2]
BMI, kg/m^2	21.6 ± 3.2 (21.3; 2.8)	21.6 ± 3.1 (21.3; 2.5)	21.5 ± 3.3 (21.3; 3.3)	0.898[2]
Women, n (%)	52 (68.4)	22 (64.7)	30 (71.4)	0.622[3]
Vitamin B-12 supplement users,[4] n (%)	53 (69.7)	22 (64.7)	31 (73.8)	0.456[3]
Duration of vegan diet, y, n (%)				0.738[3]
2 to <5	54 (71.1)	23 (67.6)	31 (73.8)	
5 to <10	17 (22.4)	8 (23.5)	9 (21.4)	
≥10	5 (6.6)	3 (8.)	2 (4.8)	

[1] Geometric mean ± SD; median; IQR in parentheses (all such values).
[2] P values between study arms were determined with the use of a general linear model univariate ANOVA test that was applied on log-transformed data.
[3] P values were determined with the use of a chi-square test.
[4] Information on the use of vitamin B-12–containing supplements was determined as yes or no.

holotranscobalamin at baseline. The mean 4cB-12 score for the entire study population was in the negative range and was lower in vegans who did not use vitamin B-12 supplements than in those who reported the use of supplements (mean 4cB-12: −1.13 compared with −0.44, respectively; $P < 0.001$) (Table 2).

Between-group differences in vitamin B-12 markers at baseline and 12 wk

Baseline concentrations of vitamin B-12 and holotranscobalamin were higher, and tHcy concentrations were lower, in the vitamin B-12 arm than in the placebo arm. However, the mean baseline MMA concentration did not differ significantly between study arms (Table 3).

In addition, the amounts of toothpaste that were returned at the end of the study period did not differ between study arms (geometric mean: 160 g in the placebo arm compared with 154 g in the treatment arm) or according to the type of toothbrush used (geometric mean: 163 g for the manual brush compared with 145 g for the electrical brush).

At 12 wk, concentrations of vitamin B-12 and holotranscobalamin were higher, concentrations of MMA were lower, and concentrations of tHcy tended to be lower in the vitamin B-12 arm than in the placebo arm after adjustment for baseline concentrations of the same markers (Table 3). Changes (12-wk minus baseline values) in serum vitamin B-12 (mean: 81 compared with −27 pmol/L, respectively), holotranscobalamin (26 compared with −5 pmol/L, respectively), tHcy (−0.7 compared with 2.0 μmol/L, respectively), and MMA (−0.169 compared with −0.036 μmol/L, respectively) were all significantly greater in the vitamin B-12 arm than in the placebo arm after adjustment for baseline concentrations (Table 3). In addition, the change in combined vitamin B-12 indicator (cB-12)

TABLE 2
Baseline characteristics and vitamin B-12 markers of vegans according to supplement use[1]

		Use of vitamin B-12–containing supplements		
	All subjects	No	Yes	P[2]
n	76	23	53	—
Age,[3] y	29.4 ± 7.1 (29.0; 10.0)	30.2 ± 5.8 (30.0; 8.0)	29.1 ± 7.6 (29.0; 11.0)	0.682
Women, n (%)	52 (68.4)	19 (82.6)	33 (62.3)	0.109[4]
Duration of vegan diet, y, n (%)				0.652[4]
2 to <5	54 (71.1)	18 (78.3)	36 (67.9)	
5 to <10	17 (22.4)	4 (14.4)	13 (24.5)	
≥10	5 (6.6)	1 (1.3)	4 (7.5)	
Vitamin B-12,[3] pmol/L	174 ± 134 (165; 151)	135 ± 91 (135; 114)	194 ± 143 (206; 167)	0.019
Holotranscobalamin,[3] pmol/L	29 ± 34 (25; 43)	22 ± 28 (21; 28)	33 ± 35 (28; 48)	0.050
tHcy,[3] μmol/L	11.7 ± 7.3 (11.1; 4.8)	15.6 ± 10.2 (14.3; 5.5)	10.3 ± 4.5 (10.4; 3.3)	<0.001
MMA,[3] μmol/L	0.327 ± 0.585 (0.272; 0.362)	0.438 ± 0.597 (0.371; 0.512)	0.288 ± 0.578 (0.231; 0.275)	0.039
4cB-12[5]	−0.67 ± 1.01 (−0.59; 1.43)	−1.13 ± 0.96 (−1.25; 1.25)	−0.44 ± 0.96 (−0.45; 1.41)	0.005
tHcy concentration ≥12.0 μmol/L, n (%)	28 (36.8)	15 (65.2)	13 (24.5)	0.002[4]
MMA concentration >0.270 μmol/L, n (%)	38 (50.0)	17 (73.9)	21 (39.6)	0.012[4]

[1] MMA, methylmalonic acid; tHcy, total homocysteine; 4cB-12, 4 markers combined vitamin B-12 indicator.
[2] P values for comparisons between the groups were determined with the use of a general linear model univariate ANOVA test that was applied on the log-transformed data, except for 4cB-12 (nonlog data).
[3] All values are geometric means ± SDs (medians; IQRs), unless otherwise indicated.
[4] P values were determined with the use of a chi-square test (for categorical variables).
[5] All values are means ± SDs (medians; IQRs). Scores were calculated from holotranscobalamin, vitamin B-12, tHcy, MMA, and age (15).

TABLE 3
Vitamin B-12 markers in vegans at baseline and after 12 wk of intervention with a placebo or vitamin B-12–fortified toothpaste[1]

	Baseline			12 wk			Changes (12 wk − baseline)			*P*-within subject change[2]	
	Placebo (n = 34)	Vitamin B-12 (n = 42)	P^3	Placebo (n = 30)	Vitamin B-12 (n = 36)	P^3	Placebo	Vitamin B-12	P^4	Placebo (n = 30)	Vitamin B-12 (n = 36)
Vitamin B-12, pmol/L	149 ± 126 (147; 132)	197 ± 137 (204; 164)	0.048	134 ± 95 (118; 95)	279 ± 134 (310; 162)	<0.001	−27 ± 64 (−9; 54)	81 ± 135 (72; 136)	<0.001	0.081	0.001
Holotranscobalamin, pmol/L	24 ± 31 (21; 37)	35 ± 35 (34; 49)	0.044	22 ± 25 (22; 24)	64 ± 34 (68; 48)	<0.001	−5 ± 17 (−2; 16)	26 ± 34 (23; 43)	<0.001	0.248	<0.001
tHcy, μmol/L	13.3 ± 8.2 (12.2; 5.0)	10.6 ± 6.2 (10.3; 3.4)	0.020	14.1 ± 11.5 (13.9; 11.4)	9.7 ± 6.4 (9.1; 3.8)	0.051	2.0 ± 5.6 (0.8; 3.7)	−0.7 ± 4.4 (−0.9; 2.9)	0.043	0.264	0.058
MMA, μmol/L	0.358 ± 0.771 (0.253; 0.416)	0.303 ± 0.361 (0.301; 0.326)	0.386	0.378 ± 0.807 (0.285; 0.391)	0.212 ± 0.123 (0.193; 0.164)	0.001	−0.036 ± 0.544 (−0.002; 0.298)	−0.169 ± 0.340 (−0.067; 0.267)	0.045	0.960	0.001
4cB-12[5]	−0.90 ± 1.08 (−0.74; 1.40)	−0.44 ± 0.91 (−0.49; 1.43)	0.049	−1.04 ± 1.02 (−0.88; 1.47)	0.17 ± 0.62 (0.18; 1.01)	<0.001	−0.11 ± 0.50 (−0.19; 0.65)	0.61 ± 0.65 (0.60; 0.94)	<0.001	0.221	<0.001
tHcy concentration ≥12.0 μmol/L, *n* (%)	18 (52.9)	10 (23.8)	0.016[6]	19 (55.9)	7 (16.7)	<0.001[6]	—	—	—	—	—
MMA concentration >0.270 μmol/L, *n* (%)	16 (47.1)	22 (52.4)	0.818[6]	17 (50.0)	10 (23.8)	<0.001[6]	—	—	—	—	—

[1] All values are geometric means ± SDs (medians; IQRs) unless otherwise indicated. The intention-to-treat population consisted of 34 subjects in the placebo arm and 42 subjects in the vitamin B-12 toothpaste arm. MMA, methylmalonic acid; tHcy, total homocysteine; 4cB-12, 4 markers combined vitamin B-12 indicator.
[2] Calculated with the use of a repeated-measures general linear model ANOVA test that was applied on available data at both time points (n = 30 in the placebo group; n = 36 in the vitamin B-12 group).
[3] Calculated with the use of general linear model univariate ANOVA test adjusted for baseline concentrations of the same markers. Except for 4cB-12, the test was applied on log-transformed data.
[4] Calculated with the use of a general linear model univariate ANOVA test that was adjusted for baseline concentrations of the same marker.
[5] Calculated from holotranscobalamin, vitamin B-12, tHcy, MMA, and age (15).
[6] Calculated with the use of a chi-square test.

during the study period was significantly different between vitamin B-12 and placebo arms (0.61 compared with −0.11, respectively; $P < 0.001$) (Table 3). Changes in vitamin B-12 markers (vitamin B-12, holotranscobalamin, and MMA) were more pronounced in the nonsupplement users (n = 6) than in supplement users (n = 30) when compared with the changes observed in the placebo arm (n = 30) (Supplemental Figure 3). In the vitamin B-12 arm, changes in vitamin B-12 and holotranscobalamin during the study period were significantly greater in supplement users than in nonusers, whereas changes in MMA and tHcy did not differ according to supplement use. In contrast, in the placebo arm, changes in all vitamin B-12 markers were minor and did not differ according to supplement use (data not shown).

At the end of the 12-wk intervention period, although 5 of 36 vegans in the vitamin B-12 arm still exhibited low vitamin B-12 status (cB-12 between −0.5 and −1.5), none of them exhibited a cB-12 score that was suggestive of possible or probable vitamin B-12 deficiency (Supplemental Figure 4).

Correlations between the concentrations of vitamin B-12 markers at baseline and after the use of the toothpaste are presented in Supplemental Table 1. All correlations were strong in the placebo arm and only moderate in the vitamin B-12 arm, which suggested that the concentrations were unchanged in the placebo arm. Moreover, changes in MMA and cB-12 after 12 wk of vitamin B-12 toothpaste use were strongly correlated with their corresponding baseline concentrations, whereas changes in holotranscobalamin, vitamin B-12, and tHcy showed weak to moderate correlations with their baseline concentrations (Supplemental Table 1).

Within-individual changes in vitamin B-12 markers over 12 wk

With consideration that the majority of vegans in our study cohort were taking vitamin B-12 supplements and were instructed to continue this behavior during the study period, we evaluated the within-individual changes in vitamin B-12 markers to rule out any unintended depletion or augmentation. In the 30 vegans who completed the trial in the placebo arm (per protocol), concentrations of vitamin B-12 tended to decline over the 12-wk period (the geometric mean changed from baseline to week 12 from 147 to 134 pmol/L, respectively; P = 0.081), whereas concentrations of holotranscobalamin, tHcy, and MMA did not change. In the 36 participants who completed the trial in the vitamin B-12 arm, serum vitamin B-12 concentrations increased [the geometric mean changed from baseline to week 12 from 201 to 279 pmol/L, respectively (+39%); P = 0.001] as did concentrations of holotranscobalamin [from 34 to 64 pmol/L, respectively (+85%); $P < 0.001$], whereas tHcy concentrations [from 10.5 to 9.7 μmol/L, respectively (−8%); P = 0.058] and MMA concentrations [from 0.301 to 0.212 μmol/L, respectively (−30%); P = 0.001] decreased over the 12-wk period [mean baseline values were from participants who completed the 12-wk study period (per protocol)].

Performance of vitamin B-12 markers in indicating B-12 status

ROC analyses were used to assess the performance (sensitivity and specificity) of each single marker and the 4cB-12 in discriminating between the placebo- and vitamin-treated vegans after

12 wk of intervention (placebo or treatment as the separator). The highest AUC was observed for holotranscobalamin (0.866) followed by the AUC for vitamin B-12 (0.844) and the AUC for 4cB-12 (AUC: 0.843) (**Supplemental Table 2**). Cutoffs of each marker, which corresponded to 90% sensitivity or specificity in the discrimination between the 2 intervention arms, are shown in Supplemental Table 2.

DISCUSSION

The current study tested the ability of vitamin B-12 that was delivered via toothpaste to reach the blood and the efficacy of the toothpaste in changing concentrations of vitamin B-12 markers after 12 wk of use. This study was conducted in a group of vegans in whom the deficiency was known to be prevalent. We observed significant changes in all vitamin B-12 markers during the study period in the vitamin B-12 arm compared with the placebo arm. Moreover, these changes were proportional to the baseline vitamin status and, thus, were stronger in vegans who were not taking supplements than in vegans who reported having taken vitamin B-12–containing supplements.

Improvement in vitamin B-12 status in the active-intervention arm

The cyanocobalamin-fortified toothpaste improved all vitamin B-12 markers within 12 wk of use, but the most prominent change was observed in serum concentrations of holotranscobalamin (Table 3). Holotranscobalamin is sensitive to recent and transient changes in vitamin B-12 intake (21, 23), and therefore, its increase in the vitamin B-12 arm indicated that the vitamin B-12 supplemented via toothpaste had been taken up and reached the circulation (21, 24). In a study in Indian subjects with very low vitamin B-12 status, oral cyanocobalamin (10 compared with 2 μg) was administered 3 times over 18 h, and holotranscobalamin was measured the next day (21). The mean ± SD serum holotranscobalamin concentration increased from 9 ± 7 to 54 ± 26 pmol/L in the 10-μg group and from 11 ± 9 to 36 ± 19 pmol/L in the 2-μg group (21). Although this increase in the holotranscobalamin concentration after just 3 doses of cyanocobalamin was likely transient, maintenance doses are expected to correct the vitamin B-12 status over a longer time period. In another study that used oral cyanocobalamin (400 μg/d), the concentration of holotranscobalamin increased within a few days of treatment initiation, and the extent of this change from the baseline concentration (+54%) exceeded the change in total vitamin B-12 (+28%) during the same period although the vitamin B-12 concentration continued to increase during the study period (~12 wk) (18). Therefore, a change in the holotranscobalamin concentration is a better indicator of a recent change in vitamin B-12 intake, whereas a change in the vitamin B-12 concentration indicates the accumulation of the vitamin over a longer period of supplementation (19, 20, 24).

Concentrations of vitamin B-12 and MMA have shown high intraclass correlation coefficients on the retesting of the same individuals who were not treated with the vitamin (25). This finding is in line with the strong correlations that we observed between baseline and 12-wk concentrations of these markers in the placebo arm (Supplemental Table 1). However, even with the significant changes that were shown in holotranscobalamin and vitamin B-12 concentrations in the vitamin B-12 arm of our study, the metabolic marker MMA showed a much smaller change over the 12-wk period (Table 3). A delayed and slower response of MMA was expected because this marker is a downstream product of the mitochondrial vitamin B-12–dependent reaction. Moreover, the relatively low baseline MMA concentration in the vitamin B-12 arm of our study would have also resulted in a lower margin for MMA reduction as has been shown in an earlier intervention study (26).

Significant changes in serum vitamin B-12 and MMA concentrations occur when therapeutic doses of cyanocobalamin (>500 μg/d) are provided for a sufficient duration of time and when the baseline vitamin B-12 status is low (23). However, the magnitudes of these changes are smaller when the vitamin B-12 status at baseline is normal or when a low supplementation dose is used (23). The effectiveness of holotranscobalamin in the assessment of the supplementation response could not be evaluated because of the limited number of studies on this subject (23). Our results showed the superiority of holotranscobalamin over MMA in reflecting recent changes in vitamin B-12 intake. The 4cB-12 score also increased in response to increased vitamin B-12 intake; however, changes in vitamin B-12 concentrations (>150 pmol/L; 90% sensitivity) and holotranscobalamin concentrations (>28 pmol/L) were at least as efficient as the 4cB-12 (greater than −0.64) in stratifying vegans according to their treatment allocation or indicating vitamin B-12 adequacy in our study settings.

Baseline vitamin B-12 status influences the response to supplementation

We observed large interindividual variations (i.e., high SDs) in the vitamin B-12–status markers at baseline, but these variations decreased after 12 wk of supplementation in the vitamin B-12 arm (Table 3). Variations at baseline may have reflected the differences in the overall duration of the vegan diet in addition to the dose and frequency of vitamin B-12–supplement use, if any. The percentage of vitamin B-12 users in our study population was higher than that in previous studies (7, 9). This difference could have been due to a higher awareness of risk of deficiency in young vegans who were more likely to participate in the current web-based recruitment process. Similar to earlier studies (23), vegans who used vitamin B-12–containing supplements had a better vitamin B-12 status at baseline and, thus, a less-prominent response to vitamin B-12 was more likely than in participants who were not using such supplements (Supplemental Figure 3).

Implications for prevention and future studies

The mechanisms of vitamin B-12 absorption via the mucosal barrier are currently not known, to our knowledge, but the toothpaste-based route described in this study may be a promising vitamin-delivering approach for at-risk groups such as vegans or the elderly. However, we do not know whether this administration route is independent of intrinsic factors and whether it could be used effectively in patients with malabsorption disorders. Moreover, we were unable to estimate the amount of absorbed vitamin after the intervention. The estimated daily amount of vitamin B-12 that was delivered into the oral cavity was ~120% higher in vegans who used the manual toothbrush than in vegans who used

the electric toothbrush (290 compared with 130 μg, respectively). However, this difference did not translate into differences in the changes of any of the vitamin B-12–status markers, especially of holotranscobalamin, which is sensitive to minor and recent changes in vitamin intake (as low as 2 μg) (21). With consideration of the short exposure time to the toothpaste, we speculate that the capacity of vitamin-B12 absorption via the oral mucosal membrane is limited compared with the amount that was available from the toothpaste (100 μg cyanocobalamin/g). A dose-response study that uses toothpaste as a carrier and a low dose of oral vitamin B-12 in a crossover design and measures the early changes in serum holotranscobalamin concentration (within 24 h) may help to resolve this issue.

The limitations of the current study deserve mentioning. First, we asked the study participants to report the use of vitamin B-12 supplements and to continue with the use of them through the study period. We rationalized that this method would prevent any vitamin B-12 depletion that may have counteracted any expected intervention-related augmentation. We interpreted the unchanged vitamin B-12 markers in the placebo arm as a confirmation of our rationale. Second, we did not have precise information on the dose or frequency of the use of vitamin B-12 supplements. Self-reported supplement use was associated with significantly better vitamin-status markers and may, in turn, affect the magnitude of the posttreatment changes that were caused by the active treatment in the vitamin B-12 arm. However, in contrast with the placebo arm, the changes in vitamin B-12, holotranscobalamin, and tHcy concentrations were significant in both supplement users and nonusers in the vitamin B-12 arm. Moreover, adjustment for the changes in markers for corresponding baseline concentrations presumably minimized any bias that could have been related to supplement use.

In conclusion, the current study shows that, compared with the placebo, vitamin B-12–fortified toothpaste results in significant increases in serum concentrations of holotranscobalamin and vitamin B-12 and a significant reduction in tHcy and MMA concentrations. These changes are more pronounced in vegans with lower baseline vitamin B-12 status. Therefore, the oral application of vitamin B-12 is able to reach the circulation and correct vitamin B-12 markers in vegans in whom vitamin B-12 deficiency is common.

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